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(54) Title: IMMUNOMODULATORY OLIGONUCLEOTIDES

(57) Abstract

Oligonucleotides containing unmethylated CpG dinucleotides and therapeutic utilities based on their ability to stimulate an immune response in a subject are disclosed. Also disclosed are therapies for treating diseases associated with immune system activation that are initiated by unmethylated CpG dinucleotides in a subject comprising administering to the subject oligonucleotides that do not contain unmethylated CpG sequences (i.e. methylated CpG sequences or no CpG sequence) to outcompete unmethylated CpG nucleic acids for binding. Further disclosed are methylated CpG containing dinucleotides for use in antisense therapies or as in vivo hybridization probes, and immunoinhibitory oligonucleotides for use as antiviral therapeutics.

IMMUNOMODULATORY OLIGONUCLEOTIDES

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Background of the Invention

DNA binds to cell membrane and is internalized

In the 1970's, several investigators reported the binding of high molecular weight DNA to cell membranes (Lerner, R.A., W. Meinke, and D.A. Goldstein. 1971. "Membrane-associated DNA in the cytoplasm of diploid human lymphocytes". *Proc. Natl. Acad. Sci. USA 68:1212*; Agrawal, S.K., R.W. Wagner, P.K. McAllister, and B. Rosenberg. 1975. "Cell-surface-associated nucleic acid in tumorigenic cells made visible with platinum-pyrimidine complexes by electron microscopy". *Proc. Natl. Acad. Sci. USA 72:928*). In 1985 Bennett et al. presented the first evidence that DNA binding to lymphocytes is similar to a ligand receptor interaction: binding is saturable, competitive, and leads to DNA endocytosis and degradation (Bennett, R.M., G.T. Gabor, and M.M. Merritt. 1985. "DNA binding to human leukocytes. Evidence for a receptor-mediated association, internalization, and degradation of DNA". *J. Clin. Invest. 76:2182*). Like DNA, oligodeoxyribonucleotides (ODNs) are able.to enter cells in a saturable, sequence independent, and temperature and energy dependent fashion (reviewed in Jaroszewski, J.W., and J.S. Cohen. 1991. "Cellular uptake of antisense oligodeoxynucleotides". *Advanced Drug Delivery Reviews 6:235*; Akhtar, S., Y. Shoji, and R.L. Juliano. 1992. "Pharmaceutical aspects of the biological stability and membrane transport characteristics of antisense oligonucleotides". In: Gene Regulation: Biology of Antisense RNA and DNA. R.P. Erickson, and J.G. Izant, eds. Raven Press, Ltd. New York, *pp. 133*; and Zhao, Q., T. Waldschmidt, E. Fisher, C.J. Herrera, and A.M. Krieg., 1994. "Stage specific oligonucleotide uptake in murine bone marrow B cell precursors". *Blood*, 84:3660). No receptor for DNA or ODN uptake has yet been cloned, and it is not yet clear whether ODN binding and cell uptake occurs through the same or a different mechanism from that of high molecular weight DNA.

Lymphocyte ODN uptake has been shown to be regulated by cell activation. Spleen cells stimulated with the B cell mitogen LPS had dramatically enhanced ODN uptake

(Feldbush, T.L., 1985. cited *supra*), augment murine NK activity (Koo, G.C., M.E. Jewell, C.L. Manyak, N.H. Sigal, and L.S. Wicker. 1988. "Activation of murine natural killer cells and macrophages by 8-bromoguanosine". *J. Immunol. 140:3249*), and synergize with IL-2 in inducing murine LAK generation (Thompson, R.A., and Z.K. Ballas. 1990. "Lymphokine-activated killer (LAK) cells. V. 8-Mercaptoguanosine as an IL-2-sparing agent in LAK generation". *J. Immunol. 145:3524*). The NK and LAK augmenting activities of these C8-substituted guanosines appear to be due to their induction of IFN (Thompson, R.A., et al. 1990. cited *supra*). Recently, a 5' triphosphorylated thymidine produced by a mycobacterium was found to be mitogenic for a subset of human γδ T cells (Constant, P., F. Davodeau, M.-A. Peyrat, Y. Poquet, G. Puzo, M. Bonneville, and J.-J. Fournie. 1994. "Stimulation of human γδ T cells by nonpeptidic mycobacterial ligands" *Science 264:267*). This report indicated the possibility that the immune system may have evolved ways to preferentially respond to microbial nucleic acids.

Several observations suggest that certain DNA structures may also have the potential to activate lymphocytes. For example, Bell et al. reported that nucleosomal protein-DNA complexes (but not naked DNA) in spleen cell supernatants caused B cell proliferation and immunoglobulin secretion (Bell, D.A., B. Morrison, and P. VandenBygaart. 1990. "Immunogenic DNA-related factors". *J. Clin. Invest. 85:1487*). In other cases, naked DNA has been reported to have immune effects. For example, Messina et al. have recently reported that 260 to 800 bp fragments of poly (dG)•(dC) and poly (dG•dC) were mitogenic for B cells (Messina, J.P., G.S. Gilkeson, and D.S. Pisetsky. 1993. "The influence of DNA structure on the in vitro stimulation of murine lymphocytes by natural and synthetic polynucleotide antigens". *Cell. Immunol.147:148*). Tokunaga, et al. have reported that dG•dC induces γ-IFN and NK activity (Tokunaga, S. Yamamoto, and K. Namba. 1988. "A synthetic single-stranded DNA, poly(dG,dC), induces interferon-α/β and -γ, augments natural killer activity, and suppresses tumor growth" *Jpn. J. Cancer Res. 79:682*). Aside from such artificial homopolymer sequences, Pisetsky et al. reported that pure mammalian DNA has no detectable immune effects, but that DNA from certain bacteria induces B cell activation and immunoglobulin secretion (Messina, J.P., G.S. Gilkeson, and D.S. Pisetsky. 1991. "Stimulation of in vitro murine lymphocyte proliferation by bacterial DNA". *J. Immunol. 147:1759*). Assuming that these data did not result from some unusual contaminant, these studies suggested that a particular structure or other characteristic of bacterial DNA renders it capable of triggering B cell activation. Investigations of mycobacterial DNA sequences have demonstrated that ODN which contain certain palindrome sequences can activate NK cells (Yamamoto, S., T. Yamamoto, T. Kataoka, E. Kuramoto, O. Yano, and T. Tokunaga. 1992. "Unique palindromic sequences in synthetic oligonucleotides are required to induce INF and augment INF-mediated natural killer activity". *J. Immunol. 148:4072;* Kuramoto, E., O.

The transcriptional activity of the CRE is increased during B cell activation (Xie, H. T.C. Chiles, and T.L. Rothstein: "Induction of CREB activity via the surface Ig receptor of B cells". *J. Immunol. 151*:880, 1993.). CREB/ATF proteins appear to regulate the expression of multiple genes through the CRE including immunologically important genes such as fos, jun B, Rb-1, IL-6, IL-1 (Tsukada, J., K. Saito, W.R. Waterman, A.C. Webb, and P.E. Auron: "Transcription factors NF-IL6 and CREB recognize a common essential site in the human prointerleukin 1β gene". *Mol. Cell. Biol. 14*:7285, 1994; Gray, G.D., O.M. Hernandez, D. Hebel, M. Root, J.M. Pow-Sang, and E. Wickstrom: "Antisense DNA inhibition of tumor growth induced by c-Ha-ras oncogene in nude mice". *Cancer Res. 53*:577, 1993), IFN-β (Du, W., and T. Maniatis: "An ATF/CREB binding site protein is required for virus induction of the human interferon B gene". *Proc. Natl. Acad. Sci. USA 89*:2150, 1992), TGF-β1 (Asiedu, C.K., L. Scott, R.K. Assoian, M. Ehrlich: "Binding of AP-1/CREB proteins and of MDBP to contiguous sites downstream of the human TGF-B1 gene". *Biochim. Biophys. Acta 1219*:55, 1994.), TGF-β2, class II MHC (Cox, P.M., and C.R. Goding: "An ATF/CREB binding motif is required for aberrant constitutive expression of the MHC class II DRa promoter and activation by SV40 T-antigen". *Nucl. Acids Res. 20*:4881, 1992.), E-selectin, GM-CSF, CD-8α, the germline Igα constant region gene, the TCR Vβ gene, and the proliferating cell nuclear antigen (Huang, D., P.M. Shipman-Appasamy, D.J. Orten, S.H. Hinrichs, and M.B. Prystowsky: "Promoter activity of the proliferating-cell nuclear antigen gene is associated with inducible CRE-binding proteins in interleukin 2-stimulated T lymphocytes". *Mol. Cell. Biol. 14*:4233, 1994.). In addition to activation through the cAMP pathway, CREB can also mediate transcriptional responses to changes in intracellular Ca^{++} concentration (Sheng, M., G. McFadden, and M.E. Greenberg: "Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB". *Neuron 4*:571, 1990).

The role of protein-protein interactions in transcriptional activation by CREB/ATF proteins appears to be extremely important. Activation of CREB through the cyclic AMP pathway requires protein kinase A (PKA), which phosphorylates $CREB^{341}$ on ser^{133} and allows it to bind to a recently cloned protein, CBP (Kwok, R.P.S., J.R. Lundblad, J.C. Chrivia, J.P. Richards, H.P. Bachinger, R.G. Brennan, S.G.E. Roberts, M.R. Green, and R.H. Goodman: "Nuclear protein CBP is a coactivator for the transcription factor CREB". *Nature 370*:223, 1994; Arias, J., A.S. Alberts, P. Brindle, F.X. Claret, T. Smea, M. Karin, J. Feramisco, and M. Montminy: "Activation of cAMP and mitogen responsive genes relies on a common nuclear factor". *Nature 370*:226, 1994.). CBP in turn interacts with the basal transcription factor TFIIB causing increased transcription. CREB also has been reported to interact with dTAFII 110, a TATA binding protein-associated factor whose binding may

positions 282-284 near the conserved DNA-binding domain of CREB". *Proc. Natl. Acad. Sci. USA 91*:5642, 1994).

Summary of the Invention

The instant invention is based on the finding that certain oligonucleotides containing unmethylated cytosine-guanine (CpG) dinucleotides activate lymphocytes as evidenced by *in vitro and in vivo* data. Based on this finding, the invention features, in one aspect, novel immunostimulatory oligonucleotide compositions.

In a preferred embodiment, an *immunostimulatory oligonucleotide* is synthetic, between 2 to 100 base pairs in size and contains a consensus mitogenic CpG motif represented by the formula:

$$5' \quad X_1X_2CGX_3X_4 \quad 3'$$

wherein C and G are unmethylated, X_1, X_2, X_3 and X_4 are nucleotides and a GCG trinucleotide sequence is not present at or near the 5' and 3' termini.

For facilitating uptake into cells, CpG containing immunostimulatory oligonucleotides are preferably in the range of 8 to 40 base pairs in size. Prolonged immunostimulation can be obtained using stabilized oligonucleotides, particularly phosphorothioate stabilized oligonucleotides. Enhanced immunostimulatory activity has been observed where X_1X_2 is the dinucleotide GpA and/or X_3X_4 is the dinucleotide is most preferably TpC or also TpT. Further enhanced immunostimulatory activity has been observed where the consensus motif $X_1X_2CGX_3X_4$ is preceded on the 5' end by a T.

In a second aspect, the invention features useful methods, which are based on the immunostimulatory activity of the oligonucleotides. For example, lymphocytes can either be obtained from a subject and stimulated *ex vivo* upon contact with an appropriate oligonucleotide; or a non-methylated CpG containing oligonucleotide can be administered to a subject to facilitate in vivo activation of a subject's lymphocytes. Activated lymphocytes, stimulated by the methods described herein (e.g. either *ex vivo* or *in vivo*), can boost a subject's immune response. The immunostimulatory oligonucleotides can therefore be used to treat, prevent or ameliorate an immune system deficiency (e.g., a tumor or cancer or a viral, fungal, bacterial or parasitic infection in a subject. In addition, immunostimulatory oligonucleotides can also be administered as a vaccine adjuvant, to stimulate a subject's

In a sixth and final aspect, the invention features various uses for immunoinhibitory oligonucleotides. Immunoinhibitory oligonucleotides have antiviral activity, independent of any antisense effect due to complementarity between the oligonucleotide and the viral sequence being targeted.

Other features and advantages of the invention will become more apparent from the following detailed description and claims.

Detailed Description of the Invention

Definitions

As used herein, the following terms and phrases shall have the meanings set forth below:

An "oligonucleotide" or "oligo" shall mean multiple nucleotides (i.e. molecules comprising a sugar (e.g. ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a substituted pyrimidine (e.g. cytosine (C), thymine (T) or uracil (U)) or a substituted purine (e.g. adenine (A) or guanine (G)). The term "oligonucleotide" as used herein refers to both oligoribonucleotides (ORNs) and oligodeoxyribonucleotides (ODNs). The term "oligonucleotide" shall also include oligonucleosides (i.e. an oligonucleotide minus the phosphate) and any other organic base containing polymer. Oligonucleotides can be obtained from existing nucleic acid sources (e.g. genomic or cDNA), but are preferably synthetic (e.g. produced by oligonucleotide synthesis).

A"stabilized oligonucleotide" shall mean an oligonucleotide that is relatively resistant to in vivo degradation (e.g. via an exo- or endo-nuclease). Preferred stabilized oligonucleotides of the instant invention have a modified phosphate backbone. Especially preferred oligonucleotides have a phosphorothioate modified phosphate backbone (i.e. at least one of the phosphate oxygens is replaced by sulfur). Other stabilized oligonucleotides include: nonionic DNA analogs, such as alkyl- and aryl- phosphonates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Oligonucleotides which contain a diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation.

For facilitating uptake into cells, immunoinhibitory oligonucleotides are preferably in the range of 8 to 40 base pairs in size. Prolonged immunostimulation can be obtained using stabilized oligonucleotides, particularly phosphorothioate stabilized.

"Palindromic sequence" shall mean an inverted repeat (i.e. a sequence such as ABCDEE'D'C'B'A' in which A and A' are bases capable of forming the usual Watson-Crick base pairs. In vivo, such sequences may form double stranded structures.

An "oligonucleotide delivery complex" shall mean an oligonucleotide associated with (e.g. ionically or covalently bound to; or encapsulated within) a targeting means (e.g. a molecule that results in higher affinity binding to target cell (e.g. B-cell and natural killer (NK) cell) surfaces and/or increased cellular uptake by target cells). Examples of oligonucleotide delivery complexes include oligonucleotides associated with: a sterol (e.g. cholesterol), a lipid (e.g. a cationic lipid, virosome or liposome), or a target cell specific binding agent (e.g. a ligand recognized by target cell specific receptor). Preferred complexes must be sufficiently stable in vivo to prevent significant uncoupling prior to internalization by the target cell. However, the complex should be cleavable under appropriate conditions within the cell so that the oligonucleotide is released in a functional form.

An "immune system deficiency" shall mean a disease or disorder in which the subject's immune system is not functioning in normal capacity or in which it would be useful to boost a subject's immune response for example to eliminate a tumor or cancer (e.g. tumors of the brain, lung (e.g. small cell and non-small cell), ovary, breast, prostate, colon, as well as other carcinomas and sarcomas) or a viral (e.g. HIV, herpes), fungal (e.g. *Candida sp.*), bacterial or parasitic (e.g. Leishmania, Toxoplasma) infection in a subject.

A "disease associated with immune system activation" shall mean a disease or condition caused or exacerbated by activation of the subject's immune system. Examples include systemic lupus erythematosus, sepsis and autoimmune diseases such as rheumatoid arthritis and multiple sclerosis.

A "subject" shall mean a human or vertebrate animal including a dog, cat, horse, cow, pig, sheep, goat, chicken, monkey, rat, mouse, etc.

Mitogenic ODN sequences uniformly became nonstimulatory if the CpG dinucleotide was mutated (Table 1; compare ODN 1 to 1a; 3D to 3Dc; 3M to 3Ma; and 4 to 4a) or if the cytosine of the CpG dinucleotide was replaced by 5-methylcytosine (Table 1; ODN 1b,2b,2c,3Dd, and 3Mb). In contrast, methylation of other cytosines did not reduce ODN activity (ODN 1c, 2d, 3De and 3Mc). These data confirmed that a CpG motif is the essential element present in ODN that activate B cells.

In the course of these studies, it became clear that the bases flanking the CpG dinucleotide played an important role in determining the B cell activation induced by an ODN. The optimal stimulatory motif was determined to consist of a CpG flanked by two 5' purines (preferably a GpA dinucleotide) and two 3' pyrimidines (preferably a TpT or TpC dinucleotide). Mutations of ODN to bring the CpG motif closer to this ideal improved stimulation (e.g. compare ODN 2 to 2e; 3M to 3Md) while mutations that disturbed the motif reduced stimulation (e.g. compare ODN 3D to 3Df; 4 to 4b, 4c and 4d). On the other hand, mutations outside the CpG motif did not reduce stimulation (e.g. compare ODN 1 to 1d; 3D to 3Dg; 3M to 3Me).

Of those tested, ODNs shorter than 8 bases were non-stimulatory (e.g. ODN 4e). Among the forty-eight 8 base ODN tested, the most stimulatory sequence identified was TCAACGTT (ODN 4) which contains the self complementary "palindrome" AACGTT. In further optimizing this motif, it was found that ODN containing Gs at both ends showed increased stimulation, particularly if the the ODN were rendered nuclease resistant by phosphorothioate modification of the terminal internucleotide linkages. ODN 1585 (5' GGGGTCAACGTTCAGGGGGG 3' (SEQ ID NO:1)), in which the first two and last five internucleotide linkages are phosphorothioate modified caused an average 25.4 fold increase in mouse spleen cell proliferation compared to an average 3.2 fold increase in proliferation induced by ODN 1638, which has the same sequence as ODN 1585 except that the 10 Gs at the two ends are replaced by 10 As. The effect of the G-rich ends is *cis*; addition of an ODN with poly G ends but no CpG motif to cells along with 1638 gave no increased proliferation.

Other octamer ODN containing a 6 base palindrome with a TpC dinucleotide at the 5' end were also active if they were close to the optimal motif (e.g. ODN 4b,4c). Other dinucleotides at the 5' end gave reduced stimulation (eg ODN 4f; all sixteen possible dinucleotides were tested). The presence of a 3' dinucleotide was insufficient to compensate for the lack of a 5' dinucleotide (eg. ODN 4g). Disruption of the palindrome eliminated stimulation in octamer ODN (eg., ODN 4h), but palindromes were not required in longer ODN.

4	TCAACGTT	6.1 ± 1.4	19.2 ± 5.2
4a	....GC..	1.1 ± 0.2	1.5 ± 1.1
4b	...GCGC.	4.5.± 0.2	9.6 ± 3.4
4c	...TCGA.	2.7.± 1.0	ND
4d	..TT__AA	1.3 ± 0.2	ND
4e	-...__..	1.3 ± 0.2	1.1 ± 0.5
4f	C...__..	3.9 ± 1.4	ND
4g	--..__..CT	1.4 ± 0.3	ND
4h	....__.C	1.2 ± 0.2	ND
LPS		7.8 ± 2.5	4.8 ± 1.0

' Stimulation indexes are the means and std. dev. derived from at least 3 separate experiments, and are compared to wells cultured with no added ODN. ND = not done. CpG dinucleotides are underlined. Dots indicate identity; dashes indicate deletions. Z indicates 5 methyl cytosine.)

The kinetics of lymphocyte activation were investigated using mouse spleen cells. When the cells were pulsed at the same time as ODN addition and harvested just four hours later, there was already a two-fold increase in ^{3}H uridine incorporation. Stimulation peaked at 12-48 hours and then decreased. After 24 hours, no intact ODN were detected, perhaps accounting for the subsequent fall in stimulation when purified B cells with or without anti-IgM (at a submitogenic dose) were cultured with CpG ODN, proliferationwas found to synergistically increase about 10-fold by the two mitogens in combination after 48 hours. The magnitude of stimulation was concentration dependent and consistently exceeded that of LPS under optimal conditions for both. Oligonucleotides containing a nuclease resistant phosphorothioate backbone were approximately two hundred times more potent than unmodified oligonucleotides.

Cell cycle analysis was used to determine the proportion of B cells activated by CpG-ODN. CpG-ODN induced cycling in more than 95% of B cells (Table 2). Splenic B lymphocytes sorted by flow cytometry into CD23- (marginal zone) and CD23+ (follicular) subpopulations were equally responsive to ODN- induced stimulation, as were both resting and activated populations of B cells isolated by fractionation over Percoll gradients. These studies demonstrated that CpG-ODN induce essentially all B cells to enter the cell cycle.

ODN were not found to induce purified macrophages to produce prostaglandin PGE2. In fact, no apparent direct effect of CpG ODN was detected on either macrophages or T cells. *In vivo* or in whole spleen cells, no significant increase in the following interleukins: IL-2, IL-3, IL-4, or IL-10 was detected within the first six hours. However, the level of IL-6 increased strikingly within 2 hours in the serum of mice injected with CpG ODN. Increased expression of IL-12 and interferon gamma (IFN-γ) by spleen cells was also detected within the first two hours.

To determine whether CpG ODN can cause in vivo immune stimulation, DBA/2 mice were injected once intraperitoneally with PBS or phosphorothioate CpG or non-CpG ODN at a dose of 33 mg/kg (approximately 500 μg/mouse). Pharmacokinetic studies in mice indicate that this dose of phosphorothioate gives levels of approximately 10 μg/g in spleen tissue (within the effective concentration range determined from the in vitro studies described herein) for longer than twenty-four hours (Agrawal, S. et al. (1991) *Proc. Natl. Acad. Sci. USA* 91:7595). Spleen cells from mice were examined twenty-four hours after ODN injection for expression of B cells surface activation markers Ly-6A/E, Bla-1, and class II MHC using three color flow cytometry and for their spontaneous proliferation using ^{3}H thymidine. Expression of all three activation markers was significantly increased in B cells from mice injected with CpG ODN, but not from mice injected with PBS or non-CpG ODN. Spontaneous ^{3}H thymidine incorporation was increased by 2-6 fold in spleen cells from mice injected with the stimulatory ODN compared to PBS or non-CpG ODN-injected mice. After 4 days, serum IgM levels in mice injected with CpG ODN in vivo were increased by approximately 3-fold compared to controls. Consistent with the inability of these agents to activate T cells, there was minimal change in T cell expression of the IL-2R or CD-44.

Degradation of phophodiester ODN in serum is predominantly mediated by 3' exonucleases, while intracellular ODN degradation is more complex, involving 5' and 3' exonucleases and endonucleases. Using a panel of ODN bearing the 3D sequence with varying numbers of phosphorothioate modified linkages at the 5' and 3' ends, it was empirically determined that two 5' and five 3' modified linkages are required to provide optimal stimulation with this CpG ODN.

Unmethylated CpG Containing Oligos Have NK Cell Stimulatory Activity

As described in further detail in Example 4, experiments were conducted to determine whether CpG containing oligonucleotides stimulated the activity of natural killer (NK) cells in addition to B cells. As shown in Table 3, a marked induction of NK activity among spleen cells cultured with CpG ODN 1 and 3Dd was observed. In contrast, there was relatively no induction in effectors that had been treated with non-CpG control ODN.

(1993)), and showed equivalent membrane binding, cellular uptake, efflux, and intracellular localization. This suggests that there may not be cell membrane proteins specific for CpG ODN. Rather than acting through the cell membrane, that data suggests that unmethylated CpG containing oligonucleotides require cell uptake for activity: ODN covalently linked to a solid Teflon support were nonstimulatory, as were biotinylated ODN immobilized on either avidin beads or avidin coated petri dishes. CpG ODN conjugated to either FITC or biotin retained full mitogenic properties, indicating no steric hindrance.

The optimal CpG motif (TGACGTT/C is identical to the CRE (cyclic AMP response element). Like the mitogenic effects of CpG ODN, binding of CREB to the CRE is abolished if the central CpG is methylated. Electrophoretic mobility shift assays were used to determine whether CpG ODN, which are single stranded, could compete with the binding of B cell CREB/ATF proteins to their normal binding site, the doublestranded CRE. Competition assays demonstrated that single stranded ODN containing CpG motifs could completely compete the binding of CREB to its binding site, while ODN without CpG motifs could not. These data support the conclusion that CpG ODN exert their mitogenic effects through interacting with one or more B cell CREB/ATF proteins in some way. Conversely, the presence of GCG sequences or other atypical CPG motifs near the 5' and/or 3' ends of ODN likely interact with CREB/ATF proteins in a way that does not cause activation, and may even prevent it.

The stimulatory CpG motif is common in microbial genomic DNA, but quite rare in vertebrate DNA. In addition, bacterial DNA has been reported to induce B cell proliferation and immunoglobulin (Ig) production, while mammalian DNA does not (Messina, J.P. et al., *J. Immunol. 147*:1759 (1991)). Experiments further described in Example 3, in which methylation of bacterial DNA with CpG methylase was found to abolish mitogenicity, demonstrates that the difference in CpG status is the cause of B cell stimulation by bacterial DNA. This data supports the following conclusion: that unmethylated CpG dinucleotides present within bacterial DNA are responsible for the stimulatory effects of bacterial DNA.

Teleologically, it appears likely that lymphocyte activation by the CpG motif represents an immune defense mechanism that can thereby distinguish bacterial from host DNA. Host DNA would induce little or no lymphocyte activation due to it CpG suppression and methylation. Bacterial DNA would cause selective lymphocyte activation in infected tissues. Since the CpG pathway synergizes with B cell activation through the antigen receptor, B cells bearing antigen receptor specific for bacterial antigens would receive one activation signal through cell membrane Ig and a second signal from bacterial DNA, and

succinimidyl-3-(2-pyridyldithio) propionate (SPDP). Oligonucleotides can alternatively be encapsulated in liposomes or virosomes using well-known techniques.

The present invention is further illustrated by the following Examples which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

Therapeutic Uses of Immunostimulatory Oligos

Based on their immunostimulatory properties, oligonucleotides containing at least one unmethylated CpG dinucleotide can be administered to a subject in vivo to treat an "immune system deficiency". Alternatively, oligonucleotides containing at least one unmethylated CpG dinucleotide can be contacted with lymphocytes (e.g. B cells or NK cells) obtained from a subject having an immune system deficiency ex vivo and activated lymphocytes can then be reimplanted in the subject.

Immunostimulatory oligonucleotides can also be administered to a subject in conjunction with a vaccine, as an adjuvant, to boost a subject's immune system to effect better response from the vaccine. Preferably the unmethylated CpG dinucleotide is administered slightly before or at the same time as the vaccine.

Preceding chemotherapy with an immunostimulatory oligonucleotide should prove useful for increasing the responsiveness of the malignant cells to subsequent chemotherapy. CpG ODN also increased natural killer cell activity in both human and murine cells. Induction of NK activity may likewise be beneficial in cancer immunotherapy.

Therapeutic Uses for Neutral Oligonucleotides

Oligonucleotides that are complementary to certain target sequences can be synthesized and administered to a subject in vivo. For example, antisense oligonucleotides hybridize to complementary mRNA, thereby preventing expression of a specific target gene. The sequence-specific effects of antisense oligonucleotides have made them useful research tools for the investigation of protein function. Phase I/II human trials of systemic antisense therapy are now underway for acute myelogenous leukemia and HIV.

In addition, oligonucleotide probes (i.e. oligonucleotides with a detectable label) can be administered to a subject to detect the presence of a complementary sequence based on detection of bound label. In vivo administration and detection of oligonucleotide

condition. One of ordinary skill in the art can empirically determine the effective amount of a particular oligonucleotide without necessitating undue experimentation.

The studies reported above indicate that unmethylated CpG containing oligonucleotides are directly mitogenic for lymphocytes (e.g. B cells and NK cells). Together with the presence of these sequences in bacterial DNA, these results suggest that the underrepresentation of CpG dinucleotides in animal genomes, and the extensive methylation of cytosines present in such dinucleotides, may be explained by the existence of an immune defense mechanism that can distinguish bacterial from host DNA. Host DNA would commonly be present in many anatomic regions and areas of inflammation due to apoptosis (cell death), but generally induces little or no lymphocyte activation. However, the presence of bacterial DNA containing unmethylated CpG motifs can cause lymphocyte activation precisely in infected anatomic regions, where it is beneficial. This novel activation pathway provides a rapid alternative to T cell dependent antigen specific B cell activation. However, it is likely that B cell activation would not be totally nonspecific. B cells bearing antigen receptors specific for bacterial products could receive one activation signal through cell membrane Ig, and a second from bacterial DNA, thereby more vigorously triggering antigen specific immune responses.

As with other immune defense mechanisms, the response to bacterial DNA could have undesirable consequences in some settings. For example, autoimmune responses to self antigens would also tend to be preferentially triggered by bacterial infections, since autoantigens could also provide a second activation signal to autoreactive B cells triggered by bacterial DNA. Indeed the induction of autoimmunity by bacterial infections is a common clinical observance. For example, the autoimmune disease systemic lupus erythematosus, which is: i) characterized by the production of anti-DNA antibodies; ii) induced by drugs which inhibit DNA methyltransferase (Cornacchia, E.J. et al., *J. Clin. Invest. 92*:38 (1993)); and iii) associated with reduced DNA methylation (Richardson, B., L. et al., *Arth. Rheum 35*:647 (1992)), is likely triggered at least in part by activation of DNA-specific B cells through stimulatory signals provided by CpG motifs, as well as by binding of bacterial DNA to antigen receptors.

Further, sepsis, which is characterized by high morbidity and mortality due to massive and nonspecific activation of the immune system may be initiated by bacterial DNA and other products released from dying bacteria that reach concentrations sufficient to directly activate many lymphocytes.

EXAMPLES

Example 1: Effects of ODNs on B Cell Total RNA Synthesis and Cell Cycle

B cells were purified from spleens obtained from 6-12 wk old specific pathogen free DBA/2 or BXSB mice (bred in the University of Iowa animal care facility; no substantial strain differences were noted) that were depleted of T cells with anti-Thy-1.2 and complement and centrifugation over lympholyte M (Cedarlane Laboratories, Hornby, Ontario, Canada) ("B cells"). B cells contained fewer than 1% $CD4^+$ or $CD8^+$ cells. $8x10^4$ B cells were dispensed in triplicate into 96 well microtiter plates in 100 µl RPMI containing 10% FBS (heat inactivated to 65°C for 30 min.), 50 µM 2-mercaptoethanol, 100 U/ml penicillin, 100 ug/ml streptomycin, and 2 mM L-glutamate. 20 µM ODN were added at the start of culture for 20 h at 37°C, cells pulsed with 1 µCi of ^3H uridine, and harvested and counted 4 hr later. Ig secreting B cells were enumerated using the ELISA spot assay after culture of whole spleen cells with ODN at 20 µM for 48 hr. Data, reported in Table 1, represent the stimulation index compared to cells cultured without ODN. Cells cultured without ODN gave 687 cpm, while cells cultured with 20 µg/ml LPS (determined by titration to be the optimal concentration) gave 99,699 cpm in this experiment. ^3H thymidine incorporation assays showed similar results, but with some nonspecific inhibition by thymidine released from degraded ODN (Matson. S and A.M. Krieg (1992) Nonspecific suppression of ^3H-thymidine incorporation by control oligonucleotides. *Antisense Research and Development 2*:325).

For cell cycle analysis, 2 X 10^6 B cells were cultured for 48 hr. in 2 ml tissue culture medium alone, or with 30 µg/ml LPS or with the indicated phosphorothioate modified ODN at 1 µM. Cell cycle analysis was performed as described in (Darzynkiewicz, Z. et al., *Proc. Natl. Acad. Sci. USA 78*:2881 (1981)).

To test the mitogenic effects of CpG ODN on human cells, perpheral blood monocyte cells (PBMCs) were obtained from two patients with chronic lymphocytic leukemia (CLL), a disease in which the circulating cells are malignant B cells. Cells were cultured for 48 hrs and pulsed for 4 hours with tritiated thymidine as described above.

Example 2: Effects of ODN on Production of IgM from B cells

Single cell suspensions from the spleens of freshly killed mice were treated with anti-Thyl, anti-CD4, and anti-CD8 and complement by the method of Leibson et al., *J. Exp. Med. 154*:1681 (1981)). Resting B cells (<,02% T cell contamination) were isolated from the 63 - 70% band of a discontinuous Percoll gradient by the procedure of DeFranco et al, *J. Exp. Med. 155*:1523 (1982). These were cultured as described above in 30 µM ODN or

cultured alone, over the total counts released after cell lysis in 2% acetic acid minus the ^{51}Cr cpm released when the cells are cultured alone.

Example 5: In vivo studies with CpG phosphorothioate ODN

Mice were weighed and injected IP with 0.25 ml of sterile PBS or the indicated phophorothioate ODN dissolved in PBS. Twenty four hours later, spleen cells were harvested, washed, and stained for flow cytometry using phycoerythrin conjugated 6B2 to gate on B cells in conjunction with biotin conjugated anti Ly-6A/E or anti-Ia^d (Pharmingen, San Diego, CA) or anti-Bla-1 (Hardy, R.R. et al., *J. Exp. Med. 159*:1169 (1984). Two mice were studied for each condition and analyzed individually.

Example 6 Titration of Phosphorothioate ODN for B Cell Stimulation

B cells were cultured with phosphorothioate ODN with the sequence of control ODN 1a or the CpG ODN 1d and 3Db and then either pulsed after 20 hr with ^3H uridine or after 44 hr with ^3H thymidine before harvesting and determining cpm.

Example 7 Rescue of B Cells From Apoptosis

WEHI-231 cells (5 x 10^4/well) were cultured for 1 hr. at 37 C in the presence or absence of LPS or the control ODN 1a or the CpG ODN 1d and 3Db before addition of anti-IgM (1μ/ml). Cells were cultured for a further 20 hr. before a 4 hr. pulse with 2 μCi/well ^3H thymidine. In this experiment, cells with no ODN or anti-IgM gave 90.4 x 10^3 by addition of anti-IgM. The phosphodiester ODN shown in Table 1 gave similar protection, though with some nonspecific suppression due to ODN degradation. Each experiment was repeated at least 3 times with similar results.

Example 8 In vivo induction of IL-6

DBA/2 female mice (2 mos. old) were injected IP with 500μg CpG or control phosphorothioate ODN. At various time points after injection, the mice were bled. Two mice were studied for each time point. IL-6 was measured by Elisa, and IL-6 concentration was calculated by comparison to a standard curve generated using recombinant IL-6. The sensitivity of the assay was 10 pg/ml. Levels were undetectable after 8 hr.

Example 9 Binding of B cell CREB/ATF to a radiolabelled doublestranded CRE probe (CREB)

Whole cell extracts from CH12.LX B cells showed 2 retarded bands when analyzed by EMSA with the CRE probe (free probe is off the bottom of the figure). The

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Arthur M. Krieg, M.D.

(ii) TITLE OF INVENTION: IMMUNOMODULATORY OLIGONUCLEOTIDES

(iii) NUMBER OF SEQUENCES: 27

(iv) CORRESPONDENCE ADDRESS:
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(F) ZIP: 02109-1875

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: ASCII text

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: ARNOLD, BETH E.
(B) REGISTRATION NUMBER: 35,430
(C) REFERENCE/DOCKET NUMBER: UIZ-013CP

(ix) TELECOMMUNICATION INFORMATION:
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(B) TELEFAX: (617)227-5941

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGGGTCAACG TTCAGGGGGG 20

(2) INFORMATION FOR SEQ ID NO:2:

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 13
(D) OTHER INFORMATION: "N indicates 5 methyl cytosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCTAGACGTT AGNGT 15

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCATGACGTT GAGCT 15

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATGGAAGGTC CAGCGTTCTC 20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

ATNGACTCTC GAGCGTTCTC 20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 18
(D) OTHER INFORMATION: "N indicates 5 methyl cytosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATCGACTCTC GAGCGTTNTC 20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATGGAAGGTC CAACGTTCTC 20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAGAACGCTG GACCTTCCAT 20

(2) INFORMATION FOR SEQ ID NO:14:

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 6
(D) OTHER INFORMATION: "N indicates 5 methyl cytosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GAGAANGCTG GACCTTCCAT 20

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 14
(D) OTHER INFORMATION: "N indicates 5 methyl cytosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GAGAACGCTG GACNTTCCAT 20

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GAGAACGATG GACCTTCCAT 20

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

TCCATGTNGG TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 12
(D) OTHER INFORMATION: "N indicates 5 methyl cytosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TCCATGTCGG TNCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TCCATGACGT TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TCCATGTCGG TCCTGCTGAT 20

(2) INFORMATION FOR SEQ ID NO:27:

CLAIMS

1. An oligonucleotide comprising from about 2 to about 100 nucleotides and containing at least one unmethylated CpG dinucleotide.

2. The oligonucleotide of claim 1 which contains a sequence represented by the following formula:

$$5' \quad X_1X_2CGX_3X_4 \quad 3'$$

wherein C and G are unmethylated, X_1, X_2, X_3 and X_4 are nucleotides and a GCG trinucleotide sequence is not present at or near the 5' and 3' termini.

3. The oligonucleotide of claim 2 having a phosphate backbone modification.

4. The oligonucleotide of claim 3 wherein the phosphate backbone modification is a phosphorothioate backbone modification.

5. The oligonucleotide of claim 1 comprising the following nucleotide sequence:

5' GGGGTCAACGTTGAGGGGGG 3' (SEQ ID NO:1)

6. The oligonucleotide of claim 5 having a phosphate backbone modification.

7. The oligonucleotide of claim 6 wherein the phosphate backbone modification is a phosphorothioate modification.

8. An oligonucleotide delivery complex comprising the oligonucleotide of claim 1 and a targeting means.

9. An oligonucleotide delivery complex of claim 8, wherein the targeting means is selected from the group consisting of cholesterol, virosome, liposome, lipid, and a target cell specific binding agent.

19. A method for treating a disease associated with an immune system activation in a subject comprising administering to the subject an effective amount of a neutral oligonucleotide alone or in conjunction with a pharmaceutically acceptable carrier.

20. A method of claim 19 wherein the disease associated with immune system activation is systemic lupus erythematosus.

21. A method of claim 19 wherein the disease associated with immune system activation is sepsis.

22. An improved method for performing antisense therapy comprising methylating CpG containing oligonucleotides prior to administration to a subject.

23. An improved method for in vivo diagnoses using oligonucleotide probes comprising methylating CpG containing oligonucleotides prior to administration to a subject.

24. An oligonucleotide which is capable of interfering with the activity of viral or cellular transcription factors and containing a consensus immunoinhibitory CpG motif represented by the formula:

$$5'GCGXnGCG3'$$

wherein X = a nucleotide and n = in the range of 0-50.

25. An oligonucleotide of claim 24, wherein X is a pyrimidine.

26. An oligonucleotide of claim 25, wherein Xn is a CpG dinucleotide.

27. A method for treating or preventing a viral infection in a subject comprising administering to the subject an immunoinhibitory oligonucleotide of claim 24.

INTERNATIONAL SEARCH REPORT

Inte nal Application No
PCT/US 95/01570

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF VIROLOGY, vol.46, no.1, April 1983 pages 204 - 211 R.REPASKE ET AL. 'Nucleotide Sequence of the env-Specific Segment of NFS-Th-1 Xenotropic Murine Leukemia Virus.' see the whole document	1
A	JOURNAL OF VIROLOGY, vol.53, no.2, February 1985 L.WOLFF ET AL. 'Sequence Comparisons of the Anemia- and Polycythemia-Inducing Strains of Friend' see the whole document	1
A	JOURNAL OF VIROLOGY, vol.64, no.11, November 1990 pages 5491 - 5499 A.C.MASSEY ET AL. 'Origin of Pathogenic Determinants of Recombinant Murine Leukemia Viruses : Analysis of Bxv-1-Related Xenotropic Viruses from CWD Mice.' see the whole document	1

1